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[Beta]-amyloid-mediated nitric oxide release from rat microglia by ligation of the integrin Mac-1

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β -amyloid-mediated nitric oxide release from rat microglia by
ligation of the integrin Mac-1

by

Jeffrey Lee Goodwin

A dissertation submitted to the graduate faculty
in partial fulfillment of the requirements for the degree of
DOCTOR OF PHILOSOPHY

Major: Veterinary Anatomy

Major Professor: Etsuro Uemura

Iowa State University

Ames, Iowa

1996

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TABLE OF CONTENTS

CHAPTER 1. GENERAL INTRODUCTION	1
Literature Review	1
Research Objective	6
Dissertation Organization	7
References	8
CHAPTER 2. MICROGLIAL RELEASE OF NITRIC OXIDE BY THE SYNERGISTIC ACTION OF β -AMYLOID AND IFN- γ	16
Summary	16
Introduction	17
Materials and Methods	18
Results	20
Discussion	23
Acknowledgement	27
References	28
CHAPTER 3. THE INTEGRIN MAC-1 MEDIATES MICROGLIAL RELEASE OF NITRIC OXIDE INDUCED BY β -AMYLOID	39
Summary	39
Introduction	40
Materials and Methods	41
Results	45
Discussion	47
Acknowledgement	51
References	52
CHAPTER 4. GENERAL CONCLUSIONS	62
Summary and Discussion	62
Recommendations for Future Research	65
References	66
ACKNOWLEDGMENTS	68

CHAPTER 1. GENERAL INTRODUCTION

Literature Review

Alzheimer's disease (AD) is a progressive neuronal degenerative disorder that results in a loss of higher cognitive function. The histopathologic changes of AD are relatively stereotyped; the hallmark of AD is a loss of neurons and synapses accompanied by a gradual accumulation of β -amyloid plaques, neuritic plaques, neurofibrillary tangles, and dystrophic neurites^{66,61}. Recent interest has been directed toward β -amyloid (β 1-42), the primary protein component of the neuritic plaques^{39,8}. According to the 'amyloid theory,' it is this peptide which is responsible for initiating and promoting AD. It is important to note that β -amyloid deposition can occur in the absence of neuronal degeneration in patients with AD. Non-compacted, diffuse deposits of β -amyloid are not always associated with dystrophic neurites. In contrast, the dense-core type (or matured) amyloid plaque is almost always associated with dystrophic neurites that often contain paired helical filaments or show abnormal immunoreactivity to the tau protein^{26,29}. These observations suggest that neuritic plaques progress from a diffuse inert form to a β -pleated β -amyloid plaque accompanied by dystrophic neurites. To date, however, the effects of naturally occurring β -amyloid on the cells of the central nervous system have not been determined.

When β -amyloid plaque cores, isolated from AD tissue, were injected into the hippocampus of rats, neuronal loss was observed by one month post-injection¹⁷. Furthermore, the *in vitro* study using the cerebral cortex of Alzheimer's patients as a substratum demonstrated that the survival of rat hippocampal neurons was significantly reduced in the peri-plaque region⁷. Although the brain tissue used for intrahippocampal injection or substrate for cultured cells contained all of the constituents of plaques, not just amyloid, it is suggestive that β -amyloid from AD tissue has neurotoxic properties. In contrast, synthetic β -amyloid directly injected into the brain of experimental animals failed to induce consistent neurotoxic

effects^{36,51,56,48}. Since *in vitro* studies suggest that the soluble β -amyloid monomer requires conversion to a largely β -pleated sheet conformation and aggregation into a fibrillar form before it can confer neurotoxicity^{38,24}, it is unlikely that neurotoxic effects of synthetic β -amyloid would be seen immediately, if ever. Unfortunately, these studies were based on a short-term survival period (7 days and 3 months post-injection in rat and monkey experiments, respectively). A longer post-injection time period may be needed to induce specific neurodegenerative changes *in vivo*. For example, it required 4 months before specific neurodegenerative changes could be observed in the rat following transplantation of transfected PC 12 cells that produce β APP-C104 (i.e., the carboxyl-terminal 104 amino acids of the amyloid precursor protein)⁴⁵. Since the development of AD seems to follow many years of β -amyloid deposition and a prolonged preclinical period⁵, it is likely that the current experimental approach of intracerebral injection of β -amyloid does not exactly simulate the pathogenesis of β -amyloid *in vivo*.

Whether and how β -amyloid is neurotoxic under biologically relevant experimental conditions has been the subject of intense study. Studies carried out in cell culture using neurons derived from fetal rats produce a somewhat confusing picture of synthetic β -amyloid neurotoxicity. Whitson et al.⁶⁰ demonstrated that a peptide homologous to the first 28 amino acids of β -amyloid (i.e., β 1-28) induces neurotrophic activity in hippocampal neurons. Subsequently, it was shown that the full length β -amyloid peptide (β 1-42) increased neurite length and branching⁵⁹. These results contrast with research by Yankner et al.⁶⁴ who reported that fragments of β -amyloid, as well as the complete peptide sequence, β 1-40, were neurotoxic to hippocampal cells in primary culture. Whether β -amyloid exerts trophic or toxic effects on cultured neurons seems to depend on the stage of neuronal differentiation in culture⁶⁵. It was shown that β 1-40 was neurotrophic only during the early stages of differentiation (days 0-2 post-plating) and that β 1-40 induced degeneration of differentiated neurons (days 3-5 post-

plating). In contrast, a study by Pike et al.⁴⁶ showed that neurons at the early stages of differentiation undergo neurodegeneration if exposed to aggregated β -amyloid.

Obviously, most of the previous studies regarding β -amyloid and AD have been focused on the direct neuronal effects of β -amyloid; however, the morphological observation of diffuse, putatively 'early' neuritic plaques and classic plaques suggests that reactive glial cells are a prominent component of the neuritic plaques. Reactive cells associated with neuritic plaques include microglia, astrocytes, and T-cells^{49,42}. It has been suggested that glial cells surrounding neuritic plaques are involved in the removal of debris from degenerated neurites^{50,54} or in the synthesis and deposition of β -amyloid⁶¹. However, nearly all classic plaques have HLA-DR-positive microglial cells³⁰. T-cells, which are often difficult to distinguish from glial cells under routine histological stains, are also identified by immunocytochemistry⁴². Also present at high levels in the plaques are cell adhesion molecules such as β_2 integrins^{50,1}, as well as cytokines such as IL-1 and TGF- β ^{23,21,57}. It is possible that these elements of the neuritic plaque are in some way responsible for the manner in which neurons degenerate.

Recent *in vitro* studies suggest that β -amyloid, directly or indirectly, can induce oxidative injury mediated by the formation of free-radicals and hydrogen peroxide^{4,53,22}, suggesting oxidative damage as a potential cause of neuronal degeneration. Reactive microglial cells are known to release the free radical nitric oxide which has been shown to be toxic to neurons^{6,9,67}. Nitric oxide binds avidly to the iron-sulphur centers of enzymes, including enzymes involved in the mitochondrial electron transport chain, the citric acid cycle, and DNA synthesis²⁷. Nitric oxide also reacts with superoxide anions to form peroxynitrite anions that decompose to yield highly damaging hydroxyl free radicals and nitrogen dioxide³. Neurons seem to be especially vulnerable to oxidative stress and free-radical damage since they contain low levels of glutathione, a major antioxidant that is responsible for removal of cytosolic peroxides⁵⁵. Microglial release of nitric oxide accompanied by a marked reduction in neuronal

cell survival *in vitro* suggests significant implications of nitric oxide on CNS pathophysiology, given the anatomical location and abundance of microglial cells, and the wide variety of potential interactions that nitric oxide can have with cellular biochemistry. Microglial release of free radicals may also alter neuronal effects of β -amyloid, since oxidation of β -amyloid by free radicals can also generate insoluble protein aggregations¹⁵.

The mechanisms involved in microglial stimulation for release of nitric oxide are not well understood. What is known is that microglial nitric oxide synthase (NOS) appears to catalyze the same reaction as the neuronal NOS and requires nicotinamide adenine dinucleotide phosphate (NADPH) and flavin adenine dinucleotide (FAD), but it is calcium- and calmodulin-independent and must be induced by agents such as bacterial cell wall lipopolysaccharide (LPS) or interferon- γ (IFN- γ)^{67,63,12,20}. In regard to β -amyloid, it was shown that peritoneal macrophages, without priming with IFN- γ respond to β 1-40, but not to β 25-35, by releasing nitric oxide³⁴.

Whether β -amyloid is responsible for the activation of microglia and subsequent release of nitric oxide has not been elucidated. Furthermore, the mechanism by which β -amyloid-mediated release of microglial nitric oxide might occur remains unknown. Previous studies have shown that engagement of the surface receptor Mac-1 may be required for the maximum respiratory burst response of macrophages and polymorphonuclear cells^{13,44,52,16}. Mac-1 belongs to a family of adhesion molecules composed of a heterodimer between an α and β -subunit found almost exclusively on leukocytes or cells of this lineage²⁸. Each of these adhesion molecules shares an identical β subunit (CD18), and they are distinguished by their α subunits designated CD11a, CD11b, and CD11c for LFA-1a, Mac-1a, and p150,95 α , respectively. Mac-1 (CD11b/CD18) is the CR3 receptor and binds, most importantly, C3bi and CD54 (ICAM-1).

Microglial cells express Mac-1 that consists of an α_M chain noncovalently associated with a β_2 -chain²⁸. Although Mac-1 and its subunit β_2 are upregulated in reactive microglial cells

associated with neuritic plaques^{50,1}, its role in brain function is not clear. The function of β_2 -integrins and Mac-1 in cell adhesion has been demonstrated from the defects displayed by polymorphonuclear (PMN) leukocytes of children with leukocyte adhesion deficiency (LAD), cattle with LAD, or by normal PMN treated with anti- β_2 or anti-Mac-1 antibodies^{32,44,43}. PMN in LAD patients migrate poorly through tissues to sites of inflammation, and like normal PMN treated with antibodies directed against the β_2 -integrins, PMN in LAD patients attach and spread poorly *in vitro*. Numerous ligands for Mac-1 have been identified, including: C3bi, ICAM-1, coagulation factor X, and fibrinogen²⁸. The binding sites for those have been mapped to the α -chain¹¹. It has also been shown that Mac-1 adheres more rapidly and extensively on substrates coated with denatured protein than native protein¹⁰. Therefore, Mac-1 plays an important role in cell-cell and cell-matrix interactions²⁸.

Mac-1 was shown to have two binding sites, one for peptide and a second for lipids⁶², and the maximum respiratory burst response or nitric oxide release of macrophages and PMN requires the synergistic action of IFN- γ and LPS^{13,14,44,52,16}. These observations suggest that Mac-1 can mediate a co-stimulatory signal in a variety of cells. Although little is known about the mode of action of Mac-1 in response to stimulatory signals, it has been shown, in monocytes, that binding to CD11b of Mac-1 not only increases binding of ligands to CD-18, but also induces an optimal respiratory burst and release of hydrogen peroxide from macrophages by ligation of CD18^{19,14}. Recently it was reported that antibody to Mac-1 β was found to stimulate nitric oxide production and inducible nitric oxide synthase mRNA expression in alveolar macrophages²⁹. This is the first report of a connection between Mac-1 and nitric oxide release from a macrophage.

In summary, microglial cells have been shown to release nitric oxide that is toxic to neurons^{37,9}. Nitric oxide release by microglia in response to β -amyloid (β 25-35) may require, or may be enhanced by, co-exposure to any number of co-stimulatory factors, i.e. IFN- α/β , IFN- γ , TNF- α , TNF- β , or IL-1. Co-stimulatory factors are a common requirement for cells

of macrophage lineage when induction of respiratory burst activity, or nitric oxide production via the Mac-1 receptor, is necessary^{16,44}. Therefore, the Mac-1 receptor, known to be present on the cell surface of brain microglia¹, is a likely candidate through which β -amyloid can induce nitric oxide release in microglial cells. Understanding the way in which β -amyloid affects microglial release of nitric oxide should be critical for studying the role of microglial cells in the pathogenesis of neuronal degeneration in AD.

Research Objective

While previous studies on the neurodegeneration of Alzheimer's disease (AD) have focused predominantly on neuronal cells and direct β -amyloid-mediated neurotoxicity, few have sought to elucidate the role of reactive glial cells in the disease process. The purpose of the research in this dissertation is to examine the effects of β -amyloid on nitric oxide release from cultured microglia and to characterize a mechanism by which β -amyloid might act to exert such an effect. The extent to which β -amyloid affects nitric oxide release from microglia will be assessed by observing changes in microglial morphology, nitric oxide synthase activity, and nitric oxide release from microglia exposed to β -amyloid (β 25-35 and β 1-40) alone and in combination with the co-stimulatory factors IFN- α/β , IL-1 β , TNF- α , TNF- β , and IFN- γ . Once the effect of β -amyloid on nitric oxide release from microglia has been characterized, a potential receptor through which β -amyloid might act will be studied. The interaction of β -amyloid (β 25-35) and the microglial β_2 integrin receptor, Mac-1, will be studied by (1) assessing the suppressive effects of anti-Mac-1 monoclonal antibodies (mAbs) on β -amyloid-mediated release of microglial nitric oxide and (2) assaying competitive binding of biotinylated β 1-42 and anti-Mac-1 mAbs to microglial cells by immunofluorescent flow cytometry. The results of the research in this dissertation support a role for microglia in the neurodegeneration of Alzheimer's disease via nitric oxide toxicity induced by the synergistic action of β -amyloid with a co-stimulatory factor and mediated through the microglial surface receptor Mac-1.

Dissertation Organization

This dissertation was prepared in accordance with the Iowa State University Graduate College Thesis Manual for the inclusion of papers in a thesis. Following the Table of Contents and General Introduction, two papers are presented. The first paper has been published in *Brain Research* vol. 692 (1995) 207-214. The second paper is currently being submitted for publication to a journal in the field of neuroscience. After the second paper, a General Discussion section is presented which addresses the findings of both papers. Figures and tables are presented at the end of each paper. Reference sections are presented at the end of each chapter and include references pertaining to that chapter.

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CHAPTER 2. MICROGLIAL RELEASE OF NITRIC OXIDE BY THE SYNERGISTIC ACTION OF β -AMYLOID AND IFN- γ

¹A paper published in Brain Research

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Summary

Alzheimer's disease (AD) is a progressive neurodegenerative disorder characterized histopathologically by a loss of neurons and an accumulation of β -amyloid plaques, neurofibrillary tangles, dystrophic neurites, and reactive glial cells. While most previous studies on the neurodegeneration of AD have focused on neuronal cells and direct β -amyloid-mediated neurotoxicity, few have focused on the role of reactive glial cells in β -amyloid-mediated neurotoxicity. In the present study nitric oxide release from cultured rat microglia was examined by exposing the cells to synthetic β -amyloid peptides (β 25-35 and β 1-40) alone and in combination with the cytokines IFN- α/β (100 U/ml), IL-1 β (100 U/ml), TNF- α (100 U/ml), TNF- β (100 U/ml), or IFN- γ (10, 100, 500, or 1000 U/ml). Assessment of microglial release of nitric oxide was based on the colorimetric assay for nitrite in the culture medium and histochemistry for nitric oxide synthase. Of the cytokines tested, only IFN- γ (1000 U/ml) induced nitric oxide release from microglia. β 25-35 did not stimulate nitric oxide release by itself, but it did induce nitric oxide release when co-exposed with IFN- γ (100, 500, and 1000 U/ml). In contrast, β 1-40 did induce microglial release of nitric oxide by itself, and this effect was enhanced significantly by co-exposure with IFN- γ (100 U/ml). These findings warrant a further investigation into the role of microglia in the neurodegeneration of Alzheimer's disease

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via nitric oxide toxicity induced by the synergistic action of β -amyloid and a costimulatory factor.

Introduction

Alzheimer's disease (AD) is a progressive neurodegenerative disorder that results in a loss of higher cognitive function. The hallmark of AD is a loss of neurons and synapses accompanied by a gradual accumulation of diffuse β -amyloid plaques, neuritic plaques, neurofibrillary tangles, and dystrophic neurites^{44,45,48}. β -amyloid deposition can occur in the absence of neuronal degeneration in patients with AD. Non-compacted, diffuse (or early) deposits of β -amyloid are not always associated with dystrophic neurites and reactive astrocytes. In contrast, the dense-core type (or matured) neuritic plaques are almost always associated with dystrophic neurites, reactive microglia and astrocytes^{14,19}. These observations suggest that neuritic plaques progress from the diffuse inert form to the β -pleated β -amyloid plaque. To date, the effects of this naturally occurring β -amyloid protein on the cells of the central nervous system have not been determined.

Studies carried out in cell culture produce a somewhat confusing picture of synthetic β -amyloid neurotoxicity. Whitson et al. (1989)⁴³ demonstrated that a peptide homologous to the first 28 amino acids of β -amyloid (i.e., β 1-28) induced neurotrophic activity in hippocampal neurons. Subsequently, it was shown that the full length β -amyloid peptide (β 1-42) increased neurite length and branching⁴². These results contrast with research by Yankner et al. (1990)⁴⁹ who reported that fragments of β -amyloid, as well as the complete peptide sequence, β 1-40, were neurotoxic to hippocampal cells in primary culture. Whether β -amyloid exerts trophic or toxic effects on cultured neurons seems to depend on the stage of neuronal differentiation⁵⁰. It was shown that β 1-40 was neurotrophic only during the early stages of differentiation (days 0-2 post-plating) and that β 1-40 induced degeneration of differentiated

neurons (days 3-5 post-plating). In contrast, a study by Pike et al. (1993)³⁴ showed that neurons at the early stages of differentiation undergo neurodegeneration if exposed to aggregated β -amyloid. A recent report by Behl et al. (1994)³ showed that β -amyloid induced degeneration of susceptible PC12 cells and rat cortical neurons by hydrogen peroxide-mediated lipid peroxidation of cells, suggesting that oxidative damage may be a potential cause of the neuronal degeneration in AD.

Previous studies have focused mainly on the direct neuronal effects of β -amyloid and very little information is available on the glial effects of β -amyloid. Microglia cells, in their reactive form, are found closely associated with matured neuritic plaques in the brains of those with AD²⁰. *In vitro* studies reveal that β 1-40 induces proliferation of microglial cells and stimulates microglial release of the cytokine interleukin-1 (IL-1)¹. This is significant because IL-1 levels have been shown to be elevated around the neuritic plaques in AD brains¹³, and IL-1 is known to cause an increase in β -APP mRNA in glia cells and endothelial cells *in vitro*^{10,11}.

Rodent reactive microglial cells are also known to release the free radical nitric oxide which has been shown to be toxic to neurons *in vitro*^{4,6,26,51}. Nitric oxide binds to the iron-sulfur centers of enzymes, thus interfering with the functioning of those enzymes involved in the mitochondrial electron transport chain, citric acid cycle, and DNA synthesis¹⁵. Nitric oxide also reacts with super oxide anions and forms peroxynitrite anions that decompose to yield highly damaging hydroxyl free radicals and nitrogen dioxide². Microglial release of nitric oxide is accompanied by a marked reduction in neuronal cell survival *in vitro*⁶. This suggests significant implications for nitric oxide in CNS pathophysiology given the anatomical location and abundance of microglial cells^{24,27} and the wide variety of potential interactions that nitric oxide can have with cellular biochemistry. Therefore, the present study was carried out to

examine the microglial nitric oxide-releasing properties of β -amyloid and various nitric oxide synthase inducing cytokines.

Materials and Methods

Synthesis of β -amyloid and scrambled peptide: β -amyloid (β 25-35) and scrambled peptide²⁸ was synthesized by the Iowa State University Protein Facility and purified by the Microchemical Facility at the University of Minnesota. The scrambled peptide has the following sequence: H-Ile-Met-Leu-Gly-Asn-Gly-Ala-Ser-Ile-Gly-OH. Scrambled peptide, β 25-35, and β 1-40 (Bachem Bioscience Incorporated) were dissolved in sterile tissue culture water (Sigma) in a stock concentration of 1 mg/ml (pH 7.2) and aliquots were stored at -20 °C.

Microglial culture: Microglia were obtained from the hippocampi of 3-5 day old pups (Harlan Sprague-Dawley, Indianapolis, IN). The hippocampi were dissected and the cells separated by trypsinization and mechanical dissociation. The cell suspension was diluted to 5 ml with MEM culture media supplemented with 10% FBS, 40 mM glucose, 2 mM L-glutamine, 1 mM pyruvate, 14 mM NaHCO₃, 100 IU/ml penicillin, and 100 μ g/ml streptomycin. The cell suspension was added to 25 cm² tissue culture flasks and incubated at 37 °C in a humidified 5% CO₂ atmosphere. After 12-14 days in culture, microglia were harvested from the mixed glial culture by gently shaking the flasks, pouring the suspended microglia off, and replating them into 24-well Corning Cell Well plates. Purity of the cultures was established by observing positive staining with the anti-Mac-1 antibody and negative staining with anti-galactocerebroside c (for oligodendrocytes) and anti-glial fibrillary acidic protein (for astrocytes).

Nitric oxide release from microglia in response to β -amyloid was initially studied using the β 25-35 peptide. Microglia were plated into 24-well tissue culture plates coated with either

β 25-35 (50 μ g/well) or a control peptide (scrambled β 25-35; 50 μ g/well) and incubated for 4 days. On post-plating day 1, fresh media (500 μ l) alone, or media (500 μ l) containing IFN- γ (10, 100, 500, or 1000 U/ml; GIBCO BRL), IFN- α/β (100 U/ml; Lee Biomolecular Research), IL-1 β (100 U/ml; Boehringer Mannheim), TNF- α (100 U/ml; Genzyme), or TNF- β (100 U/ml; Genzyme), was added to the culture wells. On post-plating day 4, the culture supernatants were collected for assay of nitric oxide, and the cells were either fixed in 4% paraformaldehyde (15 minutes) for NADPH-diaphorase staining or resuspended in EBSS (2 ml/well) for cellular protein determination. Each experiment was performed in triplicate with 4 wells per condition per replication.

A similar protocol was used to study the effects of β 1-40 on microglial nitric oxide release. Microglia were plated into 24-well tissue culture plates coated with β 1-40 and incubated for 4 days. On post-plating day 1, fresh media (500 μ l) alone, or media (500 μ l) containing IFN- γ (100 U/ml), was added to the culture wells. On post-plating day 4, the supernatants were collected and the cells were processed for NADPH-diaphorase staining or protein determination. To examine whether nitrite accumulation in the media in response to β 1-40 was nitric oxide synthase-specific, microglia were exposed to 500 nM NG-Monomethyl-L-Arginine (NMA) (Calbiochem) to irreversibly inhibit nitric oxide synthase activity. NMA (500 nM) was added to the culture media immediately post-plating and its presence was maintained for the duration of the culture period. Each experiment was performed in triplicate with 4 wells per condition per replication.

Nitric oxide assay: Nitric oxide production was determined indirectly through the assay of nitrite (NO₂-), a metabolite of nitric oxide, based on the Griess reaction³⁷. Briefly, a sample of the supernatant from each microglia cell condition (500 μ l) was mixed with an equal volume of Griess reagent (a mixture of 0.1% N-(1-naphthyl)ethylenediamine dihydrochloride and 1% sulfanilamide in 2% phosphoric acid), and its absorbance was read on a Roy

Spectronic 601 spectrophotometer. Nitrite in cell-free medium was determined in each experiment and subtracted from the value obtained with cells. Nitrite concentrations were calculated from the absorbency values by plotting them on a standard curve established using sodium nitrite at known concentrations ranging from 1 to 125 μ M.

Nitric oxide synthase-positive microglia were demonstrated by NADPH-diaphorase histochemistry. Briefly, cells were fixed in 4% paraformaldehyde and incubated with 0.1% Triton X-100, 0.1 mg/ml nitroblue tetrazolium and 1.0 mg/ml β -NADPH in 0.1 M phosphate buffer at 37°C for 45 minutes. Cells were then examined under phase contrast and light-field illumination for positive (blue/purple) staining.

Cellular protein assay: Cellular protein concentrations in each culture well were determined using the Bio-Rad Protein Assay (microassay procedure). Protein concentrations were calculated from the absorbency values by plotting them on a standard curve using the bovine gamma globulin standard at concentrations ranging from 1 to 25 μ g/ml.

Statistical analysis: All data were analyzed using each litter of rat pups as an experimental unit. Data were analyzed for group differences by analysis of variance (ANOVA) and significant effects were further analyzed by the t-test.

Results

Purity of the microglial cultures, as determined by anti-Mac-1 immunocytochemistry, was greater than 95%. A change in morphology was observed in microglial cells cultured on β -amyloid substrate. Microglial cells changed from the round, flattened ameboid state (Figure 1A) to either an elongated, rod-shaped state or a compact, spherical, light refractive ameboid state after a few hours in culture for both β 25-35 and β 1-40. There was no difference in morphology between microglia grown on β 25-35 or β 1-40 substrate. A significant number of

microglial cells exposed to IFN- γ (100U/ml) became compact, spherical, and light refractive. Microglia grown in the presence of both IFN- γ and β -amyloid tended to assume the more spherical shape, although many rod-shaped cells were still present (Figure 1C).

NADPH-diaphorase staining revealed that morphological changes were not necessarily correlated with an increase in nitric oxide synthase activity. Microglial cells with either the flattened amoeboid shape or the rod shape were mostly negative with the NADPH-diaphorase stain in all conditions (Figure 1B). Microglial cells with the spherical shape induced by IFN- γ responded somewhat more frequently to the NADPH-diaphorase stain, usually staining a very faint blue color when exposed to IFN- γ alone. Exposure to both β -amyloid and IFN- γ dramatically increased the number and staining intensity of microglial cells positive for NADPH-diaphorase (Figure 1D). The spherical cells were the cells most intensely stained. The number of positive cells in any given well was less than half of the total number of cells in each well.

To determine whether β 25-35 alone or in combination with cytokines known to activate nitric oxide synthase in cultured glial cells³⁰, affects microglial release of nitric oxide, nitrite levels were measured in the culture media. The cytokines tested were IFN- α/β (100 U/ml), IL-1 β (100 U/ml), TNF- α (100 U/ml), TNF- β (100 U/ml), or IFN- γ at various concentrations (10, 100, 500, or 1000 U/ml). Table 1 summarizes these results. Nitrite levels in the microglial supernatant were not affected by either control peptide or β 25-35 ($F=2.658$, $df=2,24$, $P=0.091$). Of the cytokines tested, only IFN- γ (100U/ml or 500U/ml) in combination with β 25-35 elicited significant nitrite levels ($F=5.692$, $df=7,16$, $P=0.002$). None of the other cytokines induced a significant increase in nitrite levels regardless of the experimental condition ($F=1.658$, $df=3,24$, $P=0.202$).

In subsequent experiments, nitrite levels were more accurately determined by considering the possibility that cellular proliferation may have affected the levels of nitrite in the culture medium. Cellular protein content was assayed to detect cell proliferation, and microglial

release of nitric oxide was expressed as a ratio of nitrite per milligrams of microglial protein. There was no significant difference in protein concentrations among any of the experimental groups including those with the highest nitrite values, i.e., β 25-35 in combination with IFN- γ ($F=1.386$, $df=2,3$, $P=0.370$). However, a ratio of nitrite per milligram of microglial protein was significantly increased by IFN- γ (1000 U/ml) alone ($t=3.974$, $df=4$, $P=0.020$) or IFN- γ (100, 500, or 1000 U/ml) in combination with β 25-35 ($F=4.34$, $df=4,33$, $P=0.006$). This experiment shows that IFN- γ significantly induces nitric oxide release from microglial cells exposed to β 25-35, and does so in a dose dependent manner (Figure 2). Optimum levels of nitrite in microglial cultures appear to be at IFN- γ concentrations starting at 100 U/ml in combination with β 25-35.

The microglial response to β 1-40 was studied with or without co-exposure with IFN- γ to compare it with β 25-35. Nitrite was assayed in the culture media of microglial cells grown on β 1-40 for 4 days and exposed to IFN- γ (100 U/ml) for 3 days starting at post-plating day 1. Cellular protein content was also assayed. Microglial release of nitric oxide was expressed as a ratio of nitrite per milligrams of microglial protein (Figure 3). Unlike the β 25-35 condition, the cellular protein assay indicated a significant increase in protein levels in groups of microglial cells exposed to β 1-40 ($t=-6.047$, $df=4$, $p=0.0038$). However, the nitrite/protein ratio also indicated a significant increase in microglial nitric oxide release as compared with the control condition ($t=-3.727$, $df=4$, $p=0.0033$). β 1-40 co-exposed with IFN- γ (100 U/ml) elicited an even greater effect. This synergistic effect of β 1-40 and IFN- γ was significant as compared with either β 1-40 ($t=-4.250$, $df=4$, $P=0.0008$) or IFN- γ alone ($t=-7.651$, $df=4$, $p=0.0001$).

To examine whether nitrite accumulation in the media was nitric oxide synthase-specific, microglial cells were exposed to NG-Monomethyl-L-Arginine (NMA, 500 nM), a guanidino-N-substituted L-arginine analog that acts as a competitive inhibitor of nitric oxide synthase¹⁵. The effect of β 1-40 on nitrite levels in the microglial supernatant was substantially

reduced by the inclusion of NMA in the cultures. Cultures containing NMA were not significantly different from the control ($t=-0.013$, $df=4$, $P=0.98$).

Discussion

Until recently, very little research has focused on a possible role for glial cells in β -amyloid-mediated neuronal degeneration in AD. The differences in morphology of diffuse, putatively 'early' β -amyloid plaques of AD which lack reactive glia cells, and classic neuritic plaques which clearly have reactive glia cells, along with degenerating neurites, favor some kind of glial involvement. The present study shows that microglia are induced to release nitric oxide by $\beta 1-40$ alone and by the synergistic action of $\beta 25-35$ with IFN- γ .

The cellular effects of β -amyloid were studied using synthetic β -amyloid peptides ($\beta 25-35$ and $\beta 1-40$). $\beta 1-40$ is a large fragment of the 42 amino acid β -amyloid protein found in the neuritic plaques, and $\beta 25-35$ peptide, corresponding to amino acids 25-35 of the full length peptide, has been shown to be a neurotoxic portion of β -amyloid^{28,47}. The present study examined microglial release of nitric oxide by exposing microglia to $\beta 25-35$ and $\beta 1-40$ coated on to the surface of culture wells. This exposure method differs from that of previous studies. Most previous tissue culture studies have sought to determine the effects of β -amyloid by solubilizing it or suspending it in culture medium despite the fact that β -amyloid found in the AD neuritic plaques is relatively immobile and insoluble. Our alternative method not only ensures that most cells are exposed to β -amyloid, but it also prevents any possible physical cellular damage by aggregated, mobile β -amyloid.

The results of this study support the notion that β -amyloid may be responsible for activation of microglia *in vivo*. This assertion is based on both morphological observations and assay of nitric oxide release (nitric oxide being a product of an activated macrophage) from cultured microglia in response to β -amyloid. Microglial cells are known to undergo a variety of morphological transformations *in vitro* in response to various cytokines (e.g., IFN- γ and

colony stimulating factors) and other factors (e.g., LPS)^{38,39}. These changes in morphology are thought to indicate changes in the function of microglia, based on the correlation between enzymatic activity, superoxide anion formation, and proliferation with the various morphological forms of cultured microglia³⁸. It is suggested that the round amoeboid microglia seen in culture are the most active form, rod-shaped microglia are the proliferative form, and ramified microglia (a branched form not observed in the present study) are resting microglia³⁸. If these criteria are applied to the present study, it would appear that β -amyloid (β 25-35 and β 1-40) can cause both proliferation and activation of microglia, since both the amoeboid and rod-shaped forms were present in cultures exposed to β -amyloid.

To examine whether these morphological transformations induced by β -amyloid and IFN- γ reflect nitric oxide synthase activity, microglial cells were stained with a marker for NADPH-diaphorase whose enzymatic activity is accepted as a histochemical indicator of NOS activity⁴¹. Nitric oxide synthase activity did not exactly correlate with a given morphology of microglia. While it was the spherical (active) amoeboid cells in the β -amyloid condition that usually showed the most nitric oxide synthase activity, cells with identical morphology in the IFN- γ condition did not always show increased nitric oxide synthase activity. There were subjectively more spherical cells in the β -amyloid condition when IFN- γ was added, and it was these cells that showed the most nitric oxide synthase activity. These observations would suggest that while IFN- γ can activate cells (inducing the spherical amoeboid morphology), it is the synergistic action of both β -amyloid and IFN- γ which consistently induces microglial nitric oxide synthase activity.

Much remains to be learned about the mechanisms involved in the release of nitric oxide. In the peritoneal macrophage, IFN- γ , but not other cytokines, stimulated the release of nitric oxide⁴⁶, whereas in smooth muscle cells²¹ and pancreatic macrophages²³ it is interleukin-1 (IL-1) which mediates such an effect. Activation of cortical microglial cells with IFN- γ and LPS induced high levels of nitric oxide, accompanied by a marked reduction in

neuronal cell survival⁶. Therefore, it appears that maximum nitric oxide release from cells of the mononuclear phagocytic system (including microglia) depends on the synergistic action of a priming agent (e.g. IFN- α/β , IFN- γ , TNF- α/β , or IL-1 β) and a triggering agent (e.g. LPS)^{6,9,31,51}.

In the present study β -amyloid (β 25-35 peptide) is used as a triggering agent for nitric oxide release from microglia, and it is co-exposed with the priming agents IFN- α/β , IFN- γ , TNF- α , TNF- β , or IL-1 β . While β 25-35 alone did not trigger nitric oxide release from hippocampal microglia, it did so when microglia were activated with IFN- γ (100, 500, and 1000 U/ml). IFN- γ is known to activate microglia, the consequences of which would include increased IL-1 and superoxide anion release, and decreased chemotaxis⁷. In our study, IFN- γ did significantly induce nitric oxide release from microglia and this effect was greatest in the presence of β 25-35. It would appear that while IFN- γ activates hippocampal microglia, it is the synergistic action of IFN- γ and β 25-35 that results in maximum nitric oxide release. Likewise, β 25-35 alone does not induce nitric oxide release from microglia unless microglia are first activated with a factor such as IFN- γ . This is supported by the changes in microglia morphology mentioned earlier. The nitric oxide-releasing effect of β 25-35 is specific to this peptide, as a control peptide (scrambled β 25-35) coated onto culture wells in the same manner did not elicit nitric oxide release from microglia, even when co-exposed with IFN- γ .

Our morphologic observations of a subjective increase in the appearance of rod-shaped microglia in the β 25-35 conditions suggest a proliferation of microglial cells. However, cellular protein analysis detected no significant differences in microglial protein among any of the experimental conditions involving β 25-35 and the various cytokines. Therefore, nitric oxide release from microglia exposed to β 25-35 and IFN- γ , expressed as nitrite/mg protein, must be the result of increased nitric oxide synthase activity per microglia cell and can not be accounted for simply by an increase in nitric oxide-releasing microglia.

Microglia exposed to β 1-40 responded somewhat differently from those exposed to β 25-35. First, β 1-40 induced a significant nitric oxide release from microglial cells by itself. This has been seen in peritoneal macrophages where β 1-40, but not β 25-35, induced nitric oxide release²². Also, unlike β 25-35, β 1-40 did induce proliferation of microglia, as shown previously¹. Protein analysis showed a significant increase in protein content in the β 1-40 condition. However, nitric oxide release was still significant when this proliferation was taken into account by calculating nitrite/protein content. This nitric oxide-releasing effect on microglia by β 1-40 was further increased when microglia were co-exposed with IFN- γ .

The enhancing effect of IFN- γ on β -amyloid-mediated release of microglial nitric oxide suggests a possible role for T-cells at the sites of β -amyloid deposit. IFN- γ is produced predominantly by activated T-cells¹⁸. In healthy mammals, the CNS contains T-lymphocytes in such low concentrations that they are virtually undetectable by immunohistochemical methods¹⁶. In AD, T-cells are observed in the brain and are often associated with neuritic plaques^{29,35} along with HLA-DR-positive microglial cells and cytokines (IL-1, TGF- β)^{12,13,40}. HLA-DR expression is generally considered to signify the immunocompetent status of a cell³⁶, and it is involved in the presentation of antigen by activated microglia to T-cells^{5,17}. Therefore, a scenario involving microglia and T-cells could conceivably be part of the pathogenesis of neurodegeneration following β -amyloid deposition in AD.

The mechanisms involved in neuritic plaque formation and neuronal degeneration in AD are not known. In the rodent, microglial nitric oxide release has been shown to be neurotoxic *in vitro*. This suggests nitric oxide could play a role in human neurodegenerative diseases such as AD. However, it must be noted that there are interspecies differences in the production of nitrites from macrophages and microglia. For instance, it was shown that human monocyte-derived macrophages do not produce detectable levels of nitrite when exposed to concentrations of IFN- γ plus LPS which would be sufficient to cause nitrite release in the mouse or rat³².

However, human monocyte-derived macrophages infected with *Mycobacterium avium* and exposed to TNF- α were shown to release high levels of nitrite⁸. In regard to microglia specifically, it was shown that human fetal microglia did not produce detectable levels of nitrite when stimulated with various cytokines including IFN- γ and an unspecified concentration of LPS²⁵. However, a more recent study showed that human fetal and adult microglia did produce low, but significant, levels of nitrite upon stimulation with IFN- γ and LPS³³. It is evident from these studies that variations of microglial nitric oxide release between species exist, but this may be due in part to differences in the methods employed, i.e., developmental stage or age of the species used, microglial isolation procedure and stimulation protocol. Therefore, until definitive studies comparing nitrite release from human and rat microglia are done, extrapolations from the results of rodent studies must be made with caution but cannot be dismissed. Furthermore, a study involving the effects of β -amyloid on human microglia is needed to establish whether β -amyloid-induced microglial nitric oxide release has a role in Alzheimer's disease.

The present study demonstrates that β 1-40 alone is sufficient to induce microglial release of nitric oxide in the rat and that this activity can be enhanced by the synergistic action of IFN- γ *in vitro*. It is possible that β -amyloid activation of microglial cells is one mechanism responsible for neuronal degeneration in humans, and therefore the possibility of a glial role in β -amyloid-mediated neurotoxicity in AD must be seriously considered.

Acknowledgements

Special thanks to Cathy Martens for her technical support. This study was supported, in part, by a professional advancement grant from the Iowa State University Graduate College and Graduate Student Senate.

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FIGURE 1. Effects of β 25-35 co-exposed with IFN- γ on the morphology and NADPH-diaphorase staining of microglia cells *in vitro*. Control microglia at post-plating day 3 (A,B) are characterized by a round, flat, ameboid-like morphology. Microglia plated on to β 25-35 substrate and exposed to IFN- γ (100 U/ml; C,D) for 60 hours starting at post-plating day 1 were characterized by either a rod-shaped or compact, spherical morphology. Microglia were stained for NADPH-diaphorase, as a measure of nitric oxide synthase activity, on post-plating day 4 (B,D). There was little or no staining of control microglia (B), whereas β 25-35 and IFN- γ -exposed microglia exhibiting the spherical morphology showed positive staining (D).

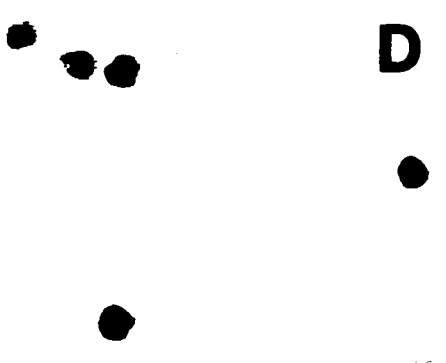
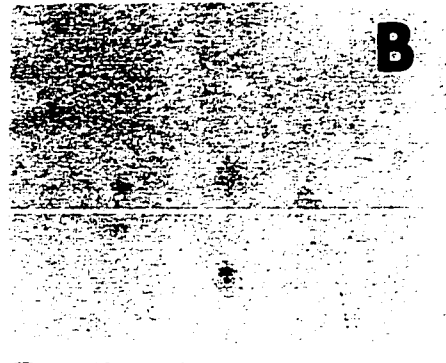


TABLE 1. Nitrite (a metabolite of nitric oxide) was assayed in supernatants from microglia exposed to various cytokines, β 25-35, or a control peptide. Microglia were exposed to β 25-35 or a control peptide for 72 hours starting on post-plating day 0, and to each cytokine for 60 hours starting at post-plating day 1. Only β 25-35 co-exposed with IFN- γ (100 U/ml) induced significant nitric oxide release from microglia. Values represent the mean \pm SEM for triplicate experiments. ** = $P < 0.02$ as compared with IFN- γ (100 U/ml) alone.

Cytokines	Concentration (U/ml)	Nitrite (μ M/50,000 cells)		
		Cytokine alone	Cytokine with control peptide	Cytokine with β 25-35
Control	N/A	0.644 \pm 0.166	0.720 \pm 0.09	0.644 \pm 0.166
IFN- α/β	100	1.613 \pm 1.064	1.765 \pm 1.04	1.936 \pm 1.274
TNF- α	100	0.587 \pm 0.050	0.740 \pm 0.15	0.625 \pm 0.069
TNF- β	100	0.606 \pm 0.119	0.775 \pm 0.14	0.587 \pm 0.050
IL-1 β	100	0.320 \pm 0.057	0.568 \pm 0.10	0.240 \pm 0.070
IFN- γ	10	0.720 \pm 0.424		1.112 \pm 0.112ns
IFN- γ	100	1.181 \pm 0.112		12.867 \pm 6.020**
IFN- γ	500	3.092 \pm 1.449		22.668 \pm 7.472**
IFN- γ	1000	6.556 \pm 1.149	5.196 \pm 4.90	14.448 \pm 2.640**

ns = not significant compared with IFN- γ (10 U/ml) alone.

** $p < 0.001$ compared with IFN- γ (100, 500 or 1000 U/ml)

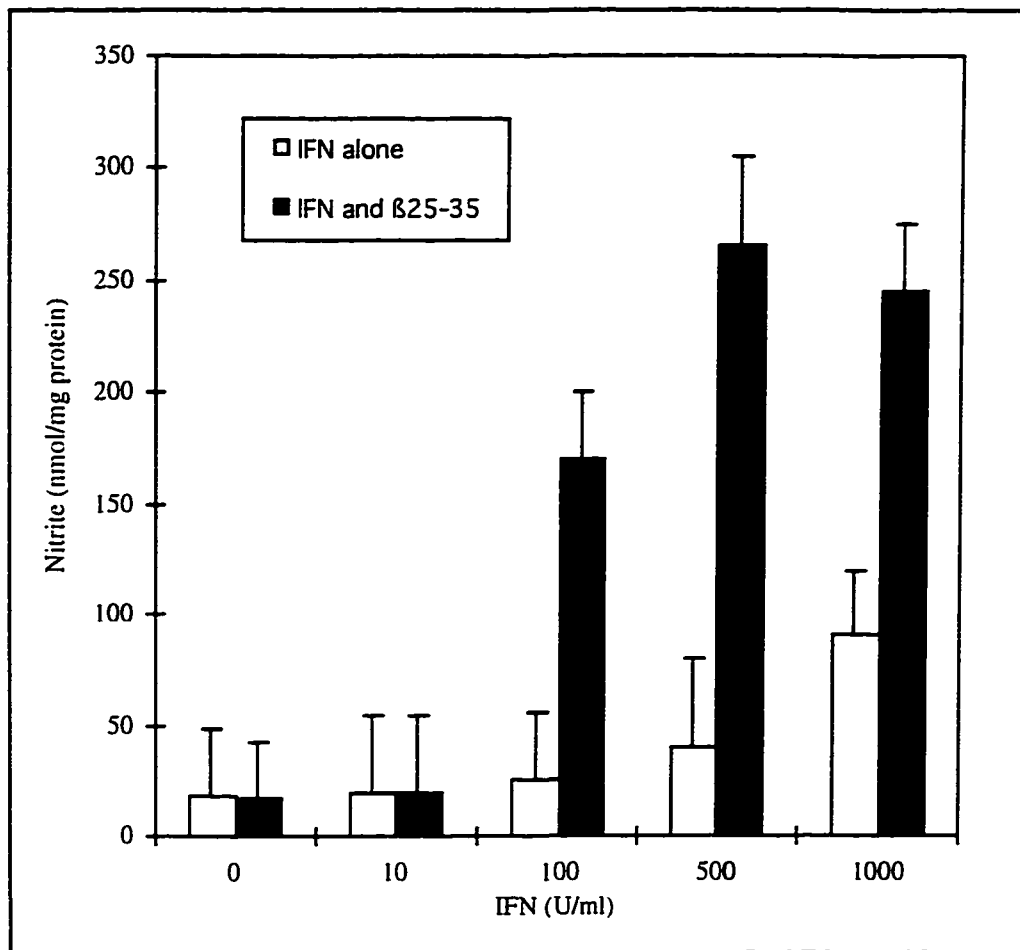


FIGURE 2. Dose response relationship for the effect of IFN- γ on nitrite release from microglia with or without co-exposure to β 25-35. Microglia were exposed to β 25-35 for 72 hours starting on post-plating day 0, and to IFN- γ for 60 hours starting at post-plating day 1. IFN- γ (1000 U/ml) and β 25-35 co-exposed with IFN- γ (100, 500, or 1000 U/ml) induced significant nitric oxide release from microglia. Values represent the mean \pm SEM for triplicate experiments.

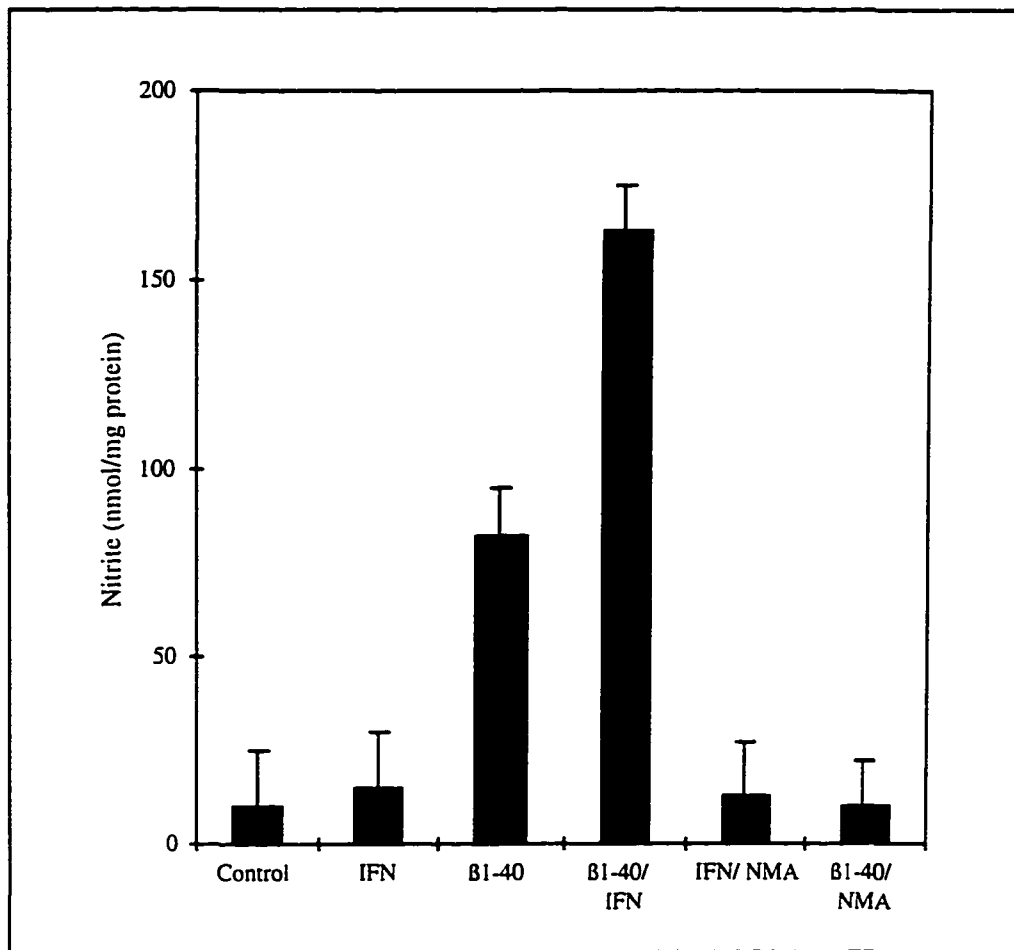


FIGURE 3. Effect of β 1-40 and IFN- γ on nitrite release from microglia. Microglia were exposed to β 1-40 for 72 hours starting on post-plating day 0, and to IFN- γ (100 U/ml) for 60 hours starting at post-plating day 1. Microglia were exposed to N^G-Monomethyl-L-Arginine (NMA) for 72 hours starting at post-plating day 0. β 1-40 induced significant nitric oxide release from microglia. The effect of β 1-40 was enhanced by co-exposure with IFN- γ and blocked by exposure to NMA. Values represent the mean \pm SEM for triplicate experiments.

CHAPTER 3: INTEGRIN MAC-1 AND β -AMYLOID IN MICROGLIAL RELEASE OF NITRIC OXIDE

A paper to be submitted to Brain Research

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Summary

The β -amyloid protein associated with Alzheimer's Disease (AD) has been well characterized biochemically; however, its primary biological function and mode of action in AD has not been determined. We have shown previously that β -amyloid (β 25-35), in combination with Interferon gamma ($\text{IFN-}\gamma$), can induce nitric oxide release from cultured hippocampal microglial cells. In the present study, binding of β -amyloid with the leukocyte integrin Mac-1, a cell surface receptor on microglia, was studied by observing (1) inhibition of β -amyloid (β 25-35)-mediated release of nitric oxide from cultured microglial cells following exposure to monoclonal antibodies against Mac-1 (anti-CD18 and anti-CD11b) and (2) competitive binding of fluorochrome-labeled β 25-35 with anti-CD18 or anti-CD11b using fluorescent flow cytometry. Wt.3 (anti-CD18 antibody) and OX42 (anti-CD11b antibody) were as effective as opsonized zymosan at inducing the release of nitric oxide from microglia. Furthermore, Wt.3 and OX42 acted synergistically to induce maximum nitric oxide release. An interaction between β -amyloid and CD18 of Mac-1 was evidenced by the suppressive action of β 25-35 on Wt.3-mediated release of nitric oxide and the synergistic action between OX42 and β 25-35 in inducing nitric oxide release from microglia. The tissue culture study was supported by competitive binding assays of fluorochrome-labeled β 25-35 and Mac-1 antibodies (Wt.3 or OX42). The majority of microglial cells (71%) did bind biotinylated β -amyloid in the presence

of Cytochalasin B, suggesting that β -amyloid binding to microglia is a receptor-mediated event. Furthermore, pre-exposure to Wt.3, but not OX42, significantly decreased binding of biotinylated β 25-35 to microglia. These findings suggest that CD18 of Mac-1 may play a role in β -amyloid-mediated release of nitric oxide.

Introduction

While the β -amyloid protein associated with Alzheimer's disease (AD) has been well characterized biochemically, its primary biological function and mode of action in AD has not been determined. β -amyloid occupies the core of neuritic plaques which are made of degenerative neurites. Microglial cells, in their reactive form, are found closely associated with matured neuritic plaques [22]; however, very little information has been available on the effects of the β -amyloid present in the neuritic plaques on microglial cells.

In the rodent, microglial cells release the free radical nitric oxide [7,8,24,42]. This may have significance in the pathogenesis of AD given that nitric oxide release from microglia is accompanied by a marked reduction in neuronal cell survival in vitro [12]. The inducible nitric oxide synthase (iNOS) found in microglia is calcium- and calmodulin-independent and must be induced by agents such as bacterial cell wall lipopolysaccharide (LPS) or IFN- γ [12,18,38,42]. We have shown that cultured microglial cells respond to IFN- γ when co-stimulated with either β 1-40 or β 25-35 to release nitric oxide, while only β 1-40 induces significant nitric oxide release from microglial cells by itself [19]. These findings were also demonstrated, in part, by Meda, et al. [26] who reported a synergistic effect between β 25-35 and IFN- γ in triggering nitric oxide and tumor-necrosis factor- α (TNF- α) release from microglia. Similarly, it was shown that rodent peritoneal macrophages without priming with IFN- γ are activated by β 1-40 but not by β 25-35 [23]. The results of these studies support the notion that β -amyloid may be responsible for activation of microglia and subsequent nitric

oxide release *in vivo*. At present, the triggering mechanisms involved in microglial stimulation for release of nitric oxide have not been documented.

Our hypothesis is that a microglial receptor through which β -amyloid might trigger nitric oxide release is the leukocyte integrin Mac-1. The Mac-1 receptor is a likely choice given that, when bound by appropriate ligands in the presence of a co-stimulatory factor such as IFN- γ , Mac-1 activates macrophages and neutrophils to produce reactive mediators including reactive oxygen intermediates and nitric oxide [13,14,16,27,31]. Coincidentally, β 25-35 in combination with IFN- γ stimulates iNOS activity and the release of nitric oxide from microglia [19]. Mac-1 belongs to a family of adhesion molecules composed of a heterodimer between an α - and β -subunit found exclusively on leukocytes or cells of this lineage. Each of these adhesion molecule shares an identical β subunit (CD18) and is distinguished by its α subunit designated CD11a, CD11b, and CD11c for LFA-1, Mac-1, and p150,95 α , respectively [21]. Microglial cells express Mac-1 and in AD, Mac-1 expression is upregulated in reactive microglial cells associated with the neuritic plaques [1,30]. Recently it was reported that antibody to CD18 was found to stimulate nitric oxide production and inducible nitric oxide synthase mRNA expression in alveolar macrophages [29]. In the present studies, we investigated a potential role for CD11b and CD18 of Mac-1 in β -amyloid-mediated release of nitric oxide from cultured hippocampal microglia.

Materials and methods

Synthesis and biotinylation of β -amyloid peptide

β -amyloid (β 25-35) was synthesized and purified by the University of Iowa Protein Structure Facility (Iowa city, IA). β 25-35 was dissolved in sterile tissue culture water (Sigma) or 0.1 M PBS (for Mac-1 binding studies) in a stock concentration of 1 mg/ml (pH 7.2) and aliquots were stored at -20 °C. β 25-35 was biotinylated according to a protocol obtained from Vector Laboratories. Briefly, β 25-35 was dissolved in 100 mM HEPES buffer, pH 8.5, at a

concentration of 2 mg/ml, then 400 μ l biotinamidocaproic acid 3-sulfo-N-hydroxysuccinimide ester (Sigma) was added, and the solution was incubated at room temperature for 2 hours. The biotinylated β 25-35 was dialyzed against 2 changes of 0.1 M PBS, pH 7.2, overnight to remove unreacted biotin ester, adjusted to a concentration of 1 mg/ml and stored at 4 °C until use.

Microglial culture

Microglia were obtained from the hippocampi of 3-5 day old rat pups (Harlan Sprague-Dawley, Indianapolis, IN). The hippocampi were dissected and the cells separated by trypsinization and mechanical dissociation. The cell suspension was diluted to 5 ml with MEM culture media supplemented with 10% FBS, 40 mM glucose, 2 mM L-glutamine, 1 mM pyruvate, 14 mM NaHCO₃, 100 IU/ml penicillin, and 100 μ g/ml streptomycin. The cell suspension was added to 25 cm² tissue culture flasks and incubated at 37 °C in a humidified 5% CO₂ atmosphere. After 12-14 days in culture, microglia were harvested from the mixed glial culture by gently shaking the flasks and pouring off the suspended microglia. The microglia were either used immediately for flow cytometry, or replated into 24-well Corning Cell well plates for the β 2 integrin blocking studies. Culture purity was established with immunocytochemistry by observing positive staining with Mac-1 antibody and negative staining with anti-galactocerebroside c for oligodendrocytes and anti-glial fibrillary acidic protein for astrocytes.

Nitric oxide blocking studies

The relationship between Mac-1 and β -amyloid-mediated nitric oxide release was studied by observing the effects of Wt.3 (anti-CD18 monoclonal antibody; Pharmingen) and OX42 (anti-CD11b monoclonal antibody; Pharmingen) on nitric oxide release from microglia. The initial study was to examine the microglial release of nitric oxide under the various control and

B25-35 conditions. Harvested microglia were pelleted, resuspended in culture media containing B25-35 (50 µg/500 µl media) at a concentration of 100,000 cells/ml, and plated onto 24-well tissue culture. On post-plating day 1, the media was replaced with fresh media (500 µl) alone, or media (500 µl) containing opsonized zymosan (200 µg/ml) or an isotype control antibody (50 µg/ml). On post-plating day 4, the culture supernatants were collected for assay of nitric oxide, and the cells were resuspended in EBSS (1 ml/well) for cellular protein determination.

To determine if there was a competitive effect between β -amyloid and Wt.3 or OX42 in mediating nitric oxide release, harvested microglia were pelleted, resuspended in 0.1 M PBS (100 µl), pH 7.2, containing B25-35 (50 µg/500 µl media), and plated onto 24-well tissue culture plates. Opsonized zymosan was used as a positive control. On post-plating day 1, fresh media (500 µl) alone, or media containing and 5 µg/ml Wt.3, OX42 or the isotype control antibody was added to the culture wells. On post-plating day 4, the culture supernatants were collected for assay of nitric oxide and cellular protein. Each experiment was repeated 3 times using quadruplicate wells.

Measurement of nitrite

Nitric oxide production was determined indirectly through the assay of nitrite (NO_2), a metabolite of nitric oxide, based on the Griess reaction [23]. Briefly, a sample of the supernatant from each microglia cell condition (500 µl) was mixed with an equal volume of Griess reagent (a mixture of 0.1% N-(1-naphthyl)ethylenediamine dihydrochloride and 1% sulfanilamide in 2% phosphoric acid), and its absorbance at 550 nm was read on a Roy Spectronic 601 spectrophotometer. Nitrite in cell-free medium was determined in each experiment and subtracted from the value obtained with cells. Nitrite concentrations were calculated from the absorbency values by plotting them on a standard curve established using sodium nitrite at known concentrations ranging from 1 to 125 µM.

Cellular protein assay

In the Mac-1 blocking studies, cellular protein concentrations in each culture well were determined using the Bio-Rad Protein Assay (microassay procedure). Protein concentrations were calculated from the absorbency values by plotting them on a standard curve using the bovine gamma globulin standard at concentrations ranging from 1 to 25 $\mu\text{g/ml}$.

Flow cytometry studies

Interactions of β -amyloid with β_2 integrins were studied by competitive binding analyses using various combinations of unlabeled and fluorochrome labeled β -amyloid and monoclonal antibodies against the α subunit of Mac 1 (CD11b) and the common β subunit (CD18) of the β_2 integrins (Table 1). Harvested microglia were pelleted and resuspended in 1 ml of 0.1 M PBS without Ca^{2+} and Mg^{2+} at a concentration of 1×10^5 cells/ml. To verify cell surface receptor-specific β -amyloid binding to the cells, microglia were pelleted, resuspended in 100 μl biotinylated β_{25-35} (1 mg/ml, pH 7.2) and incubated for 15 minutes in the presence or absence of cytochalasin B (10 $\mu\text{g/ml}$; Sigma), which decreases any microtubule-associated internalization of β_{25-35} . Cells were washed once in PBS, resuspended in 100 μl PBS containing 10 μl streptavidin (R-phycoerythrin conjugate; Sigma) and incubated for 15 minutes at room temperature. Cells were washed twice in PBS then resuspended in 500 μl sheath fluid (Isoton II; Coulter) for flow cytometric analysis.

To see if antibodies directed against the β_2 integrins could competitively inhibit the binding of biotinylated β -amyloid to microglia, microglia were pretreated with either Wt.3 or OX42. Harvested microglia were pelleted and resuspended in 1 ml PBS at 1×10^5 cells/ml. Cells were centrifuged, resuspended in 100 μl PBS containing 10 μl (50 $\mu\text{g/ml}$ stock concentration) Wt.3 or OX42 and incubated for 15 minutes at room temperature. Cells were washed once in PBS then incubated with biotinylated β_{25-35} and prepared for fluorescence analysis as described above.

Immunofluorescent analysis of the flouorochrome labeled microglia was performed in a fluorescence-activated cell sorter fluorometer (FACScan; Becton, Dickinson & Co). Values were obtained for specific fluorescence intensity (mean fluorescence channel of histogram) and percentage of cells positively labelled on at least 3 separate occasions for each condition using Cell Quest® software.

Statistical analysis

All data were analyzed for group differences by analysis of variance (ANOVA) and significant effects were further analyzed by the Tukey's B posthoc procedure.

Results

The effect of Wt. 3 and OX42 on nitric oxide release from microglia

Antibodies specific for antigen-binding sites on Mac-1 are known to elicit a number of cell responses including respiratory burst activity. Therefore, before observing the effects of these antibodies on β -amyloid mediated release of nitric oxide from microglia, it was necessary to determine what if any effect Wt.3 or OX42 had on nitric oxide release from microglia. In this experiment, nitric oxide levels were measured in the culture media of microglia exposed to Wt.3 or OX42. We first examined the microglial release of nitric oxide under the various control (opsonized zymosan, mouse IgG) and β -amyloid (β 25-35) conditions (Fig. 1). Opsonized zymosan, known to induce respiratory burst activity in phagocytic cells by binding to Mac-1, was used as a positive control. Opsonized zymosan induced a significant increase in microglial release of nitric oxide ($F=11.8$, $df=6, 21$, $p=0.0001$). There was no difference in nitric oxide levels induced by β -amyloid (β 25-35) or β -amyloid supplemented with isotype antibody control. Since isotype antibody did not induce significant levels of nitric oxide, it was used as the negative control antibody for the study of Wt.3 and OX42 (Fig. 2). Microglial release of nitric oxide was significantly increased by Wt.3, OX42, and a combination of both antibodies ($F=47.4$, $df= 3, 48$, $p=0.0001$). Both Wt.3 and OX42 are equally effective as an

inducer of nitric oxide; furthermore, a combination of Wt.3 and OX42 acted synergistically to induce significantly more nitric oxide release over Wt. 3 ($t=-2.6$, $df=6$, $p=0.012$) or OX42 alone ($t=-3.0$, $df=6$, $p=0.005$).

The effect of $\beta 25-35$ on Wt.3- and OX42-induced nitrite release from microglia

Since both Wt.3 and OX42 induced nitrite release from cultured microglia, microglial cells were first exposed to $\beta 25-35$, then they were exposed to either Wt.3, OX42, or a combination of both antibodies. $\beta 25-35$ alone did not induce significant nitrite release from microglia. If β -amyloid acts at one of the antibody binding sites, it should inhibit the nitric oxide release in response to that antibody by blocking antibody-mediated activation of Mac-1. Fig. 2 shows the effect of Wt.3, OX42 and a combination of both antibodies on β -amyloid-mediated release of nitric oxide. Nitric oxide release mediated by Wt.3 was the only condition significantly reduced by preexposing cells to $\beta 25-35$ ($t=8.40$, $df=6$, $p=0.0001$). In contrast, $\beta 25-35$ significantly increased nitric oxide release by OX42 ($t=-3.59$, $df=6$, $p=0.002$). A further increase in nitric oxide release was induced by $\beta 25-35$ with a combination of Wt.3 and OX42 ($t=-5.6$, $df=6$, $p=0.001$). As a result, there was no difference in levels of nitric oxide induced by a combination of two antibodies (Wt.3 and OX42) with or without $\beta 25-35$. There was no significant difference in cellular protein content among any of the experimental groups.

$\beta 25-35$ binding to microglia

The first step in identifying a microglial β -amyloid receptor was to demonstrate that β -amyloid binds to microglia in a cell-surface specific fashion. Cultured microglia were incubated with biotinylated $\beta 25-35$ in the presence or absence of cytochalasin B, and analyzed by a flow cytometer for the mean percent positive labeling (Fig. 3) and fluorescence intensity (MFI) of microglia with biotinylated $\beta 25-35$.

The MFI of microglia exposed to biotinylated β 25-35 was significantly higher than the biotin control ($t=-3.0$, $df=4$, $p=0.017$; data not shown) and over 70% of cultured microglia bound β 25-35 ($t=-11.19$, $df=4$, $p=0.0001$). Cytochalasin B did not reduce either the MFI of microglia binding to biotinylated β 25-35 or percent of microglia which bound biotinylated β 25-35. The flow cytometry data suggest β 25-35 binding to microglia and no internalization of β 25-35 by microglia. To demonstrate the presence of specific microglial receptors with an affinity for β -amyloid, the binding of biotinylated β 25-35 was compared with that of a biotinylated scrambled control peptide. The percentage of cells positively labeled with biotinylated scrambled peptide was not significantly different from control cells.

Competitive binding between biotinylated β 25-35 and Wt.3 and OX42

Once it was established that β -amyloid bound to cultured microglia, the next step was to confirm our tissue culture experiments which suggested that β -amyloid mediates nitric oxide release from microglia by binding to CD18 of the Mac-1 integrin. If β -amyloid binds to microglial Mac-1 at either the CD18 or CD11b site, as we hypothesized, pre-incubation with the anti-CD18 or anti-CD11b monoclonal antibody should competitively inhibit binding of biotinylated β 25-35 to cultured microglial cells. To test this, mean fluorescence intensity (MFI) and percent positive labeling of microglia with biotinylated β 25-35 were analyzed in microglial cells incubated with biotinylated β 25-35 with and without pre-exposure to Wt.3 or OX42. There were significant decreases in MFI ($F=8.73$, $df=2,6$, $p=0.017$) and percent positive labeling ($F=6.11$, $df=2,6$, $p=0.036$) of microglial cells preexposed to Wt.3 as compared with the control group antibody (Fig. 4). No such effect was observed in microglial cells preexposed to OX42.

Discussion

Microglial cell interactions with β -amyloid were studied using a synthetic β 25-35 peptide. β 25-35 peptide corresponds to amino acid 25-35 of the 42 amino acid β -amyloid protein found

in the neuritic plaques and has been shown to be a neurotoxic portion of β -amyloid [25, 40]. Our previous study demonstrated that β 25-35 stimulates nitric oxide release from microglia only when coexposed with IFN- γ [19]. In search of a cellular binding site through which β -amyloid acts to trigger nitric oxide release from microglia, we investigated the leukocyte integrin Mac-1. The respiratory burst response, which includes nitric oxide release, of macrophages and granulocytes is mediated through Mac-1. A maximum response of these cells requires the synergistic action of IFN- γ and a second ligand such as LPS [13,16,27,31]. Coincidentally, iNOS activity and the release of nitric oxide from microglia is stimulated by the synergistic action of IFN- γ and β 25-35 [19]. Given these observations and that Mac-1 can mediate a co-stimulatory signal in a variety of cells, Mac-1 is a likely candidate through which β -amyloid acts to stimulate nitric oxide release from microglia.

Mac-1 is a leukocyte integrin expressed primarily on myeloid and natural killer cells. It is responsible, in part, for myeloid cell adhesion to endothelium, neutrophil homotypic adhesion and chemotaxis, and phagocytosis of opsonized particles. It does this by binding to several cell surface and soluble ligands including iC3b, ICAM, fibrinogen, factor X, filamentous hemagglutinin, lipophosphoglycan, and LPS [2,3,4,6,34,36,37]. Mac-1 consists of a CD11b α chain noncovalently associated with a CD18 β chain. The binding sites for most of the aforementioned ligands, except for a lectin-like binding site, have been mapped to the I region of the α -chain [11]. The extracellular N-terminal domains of the α and β subunits combine to form a ligand-binding head which is ultimately connected to two cytoplasmic domains. These cytoplasmic domains are believed to interact with cytoskeletal proteins or other cytoplasmic components and enable integrins to act as signaling receptors [21]. An important feature of many integrins is that they undergo activation which is accompanied by a conformational change that is required for various ligand-binding activities. Activation of the β_2 integrins can be accomplished by solid phase reactants (e.g. fibrinogen), monoclonal antibodies, and various

inflammatory mediators such as tumor necrosis factor, C5a, platelet activating factor, or IFN- γ [21].

To demonstrate that β -amyloid interacts with Mac-1 to induce nitric oxide release from microglia we performed two studies: 1) the effects of anti-Mac-1 monoclonal antibodies (Wt.3 and OX42) on β -amyloid (β 25-35)-mediated release of nitric oxide from cultured microglial cells and 2) competitive binding of fluorochrome-labeled β 25-35 with CD18 or CD11b by fluorescent flow cytometry. The first step was to determine whether microglial nitric oxide release is mediated through Mac-1. Since it has been shown that monoclonal antibodies can act as natural ligands for phagocyte receptors associated with functional activation [20], we examined the effects of Wt.3 and OX42 alone on microglial release of nitric oxide. Both Wt.3 and OX42 were as effective as opsonized zymosan in inducing the microglial release of nitric oxide. Furthermore, Wt.3 and OX42 acted synergistically to induce maximum nitric oxide release. Although little is known about the mode of action of Mac-1 in response to stimulatory signals, it has been shown in monocytes that CD18 does not induce respiratory burst activity without the help of CD11b [17]. Furthermore, ligand binding to CD11b not only increases the binding of ligands to CD18, but it also induces an optimal respiratory burst and release of free radicals from macrophages by legation of CD18 [14,17]. Our data suggest that ligand binding to CD18 or CD11b alone can induce the release of nitric oxide in microglial cells, however, it is the combined action of both CD18 and CD11b that optimizes the microglial response.

Interaction of β -amyloid with the microglial Mac-1 was shown by the suppressive action of β 25-35 on Wt.3-mediated release of nitric oxide. β 25-35 significantly reduced nitric oxide release from microglia exposed to Wt.3, but not OX42. This suggests that β -amyloid binds to a site on CD18 either directly at the Wt.3 binding, or close enough to the Wt.3 binding site to interfere with the binding of Wt.3 by other means, e.g., steric hindrance. Since Mac-1 is capable of undergoing a conformational change upon activation by various stimuli [21], the suppressive action of β 25-35 on Wt.3-mediated release of nitric oxide could also be due to a

conformational change of Mac-1 by β 25-35 and subsequent disruption of Wt.3 binding at its site on CD18. A role for CD11b in nitric oxide release was also suggested by our study showing a significant increase in nitric oxide release by β 25-35 and OX42.

Supportive data on receptor-mediated binding of β -amyloid by microglia was obtained by competitive binding assays of fluorochrome-labeled β 25-35. The majority of microglial cells (71%) bound biotinylated β 25-35. This positive binding could not be explained by microtubule-associated internalization of β 25-35 as the binding was not affected by cytochalasin B, and binding was relatively specific as binding to the control peptide was not different from the biotin control condition. Our hypothesis that β -amyloid is a ligand for Mac-1 was supported by competitive binding studies of β -amyloid and Mac-1-specific monoclonal antibodies. Pretreatment of microglia with Wt.3, but not with OX42, significantly reduced binding of biotinylated β 25-35. However, the effect of Wt.3 was not complete.

There could be a number of explanations for the incomplete blocking effect of Wt.3 on microglial binding of β 25-35. Mac-1 is known to have numerous binding sites; therefore, β 25-35 could be binding to sites other than those blocked by Wt.3. Another explanation is that while Wt.3 is blocking one potential binding site on microglia for β 25-35, there are other binding sites available on microglia which will bind to β 25-35. Two recent reports suggest that this might be the case. Yan et al. [39] reported that β -amyloid binds with a microglia-surface receptor called RAGE (receptor for advanced glycation end products) and promotes induction of oxidative stress in microglia. An unrelated cell-surface receptor, class A SR (scavenger receptor), was also shown by El Khoury et al. [15] to interact with β -amyloid, leading to secretion of reactive oxygen species and cell immobilization. However, a signal transduction pathway through which RAGE or SR can trigger the production of reactive mediators including reactive oxygen intermediates and nitric oxide has not been identified. It is possible that RAGE and SR act to promote adhesion to β -amyloid and Mac-1 mediates release of reactive mediators.

Our study on rodent microglial cells assumes that nitric oxide could play a role in human neurodegenerative disease such as AD; however, studies from human microglial cells are not consistent in regard to nitric oxide release *in vitro*. It was shown that human fetal microglia did not produce detectable levels of nitric oxide when stimulated with various cytokines including IFN- γ and an unspecified concentration of LPS [24]. However, two more recent studies showed that human fetal and adult microglia produce low, but significant, levels of nitric oxide upon stimulation with IFN- γ or TNF- α and LPS [29,10]. In this respect, the presence of reactive microglia in neuritic plaques can not be ignored considering their upregulation of Mac-1 and its subunit CD18 in AD [1,30]. Until definitive studies comparing nitric oxide release from human and rat microglia are done, extrapolations from the results of studies on rodent must be made with caution but cannot be dismissed.

Acknowledgements

Special thanks to Cathy Martens (Department of Veterinary Anatomy) and Bruce Pesch (National Animal Disease Center) for their technical support. This study was supported by a professional advancement grant from the Iowa State University Graduate College, Graduate Student Senate, and Department of Veterinary Anatomy.

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Table 1. List of reagents used for tissue culture and flow-cytometric analysis of β -amyloid binding to microglial cells.

Reagents used	Unlabeled	PE-conjugated	Suppliers
anti-rat CD18 mAb	Wt.3 (IgG ₁)		Pharmingen
anti-rat CD11b mAb	OX42(IgG _{2a})		Pharmingen
Isotype control	Unrelated mouse IgG		Caltag
b-amyloid (25-35)	b25-35	Biotinylated b25-35	Univ. of Iowa
Scrambled peptide		Biotinylated peptide	Univ. of Iowa

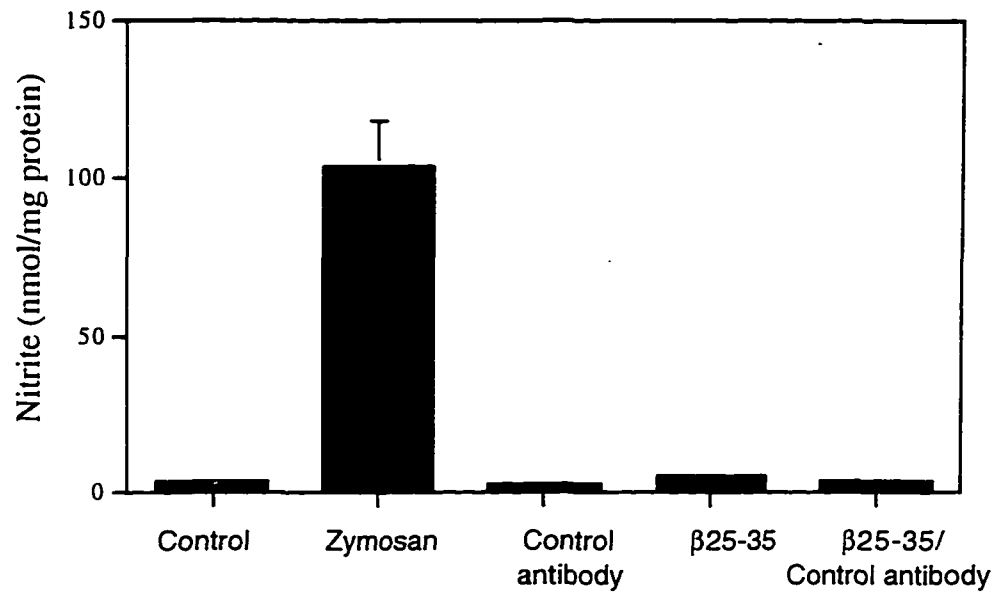


Fig. 1. The effects of β 25-35, opsonized zymosan, and isotype control antibody on nitric oxide release from cultured microglial cells. Cells were exposed to each factor for 72 hrs starting at post-plating day 0. Opsonized zymosan induced significant release of nitric oxide. Each bar represents mean values \pm SEM for nitrite/mg protein for triplicate experiments.

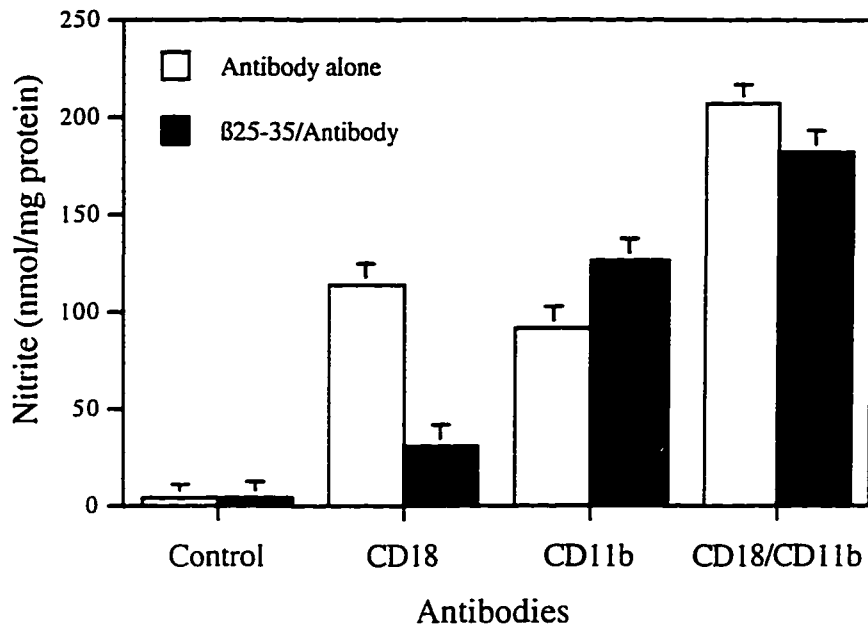


Fig. 2. The effects of Wt.3 (anti-CD18 antibody) and/or OX42 (anti-CD11b antibody) on nitric oxide release from cultured microglial cells. Microglial cells were exposed to β 25-35 for 24 hrs before exposing to Wt.3 and/or OX42. Nitrite levels were analyzed at post-plating day 4. β 25-35 alone did not stimulate microglial cells to release nitric oxide. Wt.3 and OX42 each induced significant nitric oxide release from cultured microglia, but maximum nitric oxide release was induced by the combination of Wt.3 and OX42. Nitric oxide release mediated by Wt.3 was significantly reduced by coexposing cells to β 25-35. In contrast, β 25-35 significantly increased nitric oxide release by OX42 and a combination of Wt.3 and OX42. Each bar represents mean values \pm SEM for nitrite/mg protein for triplicate experiments.

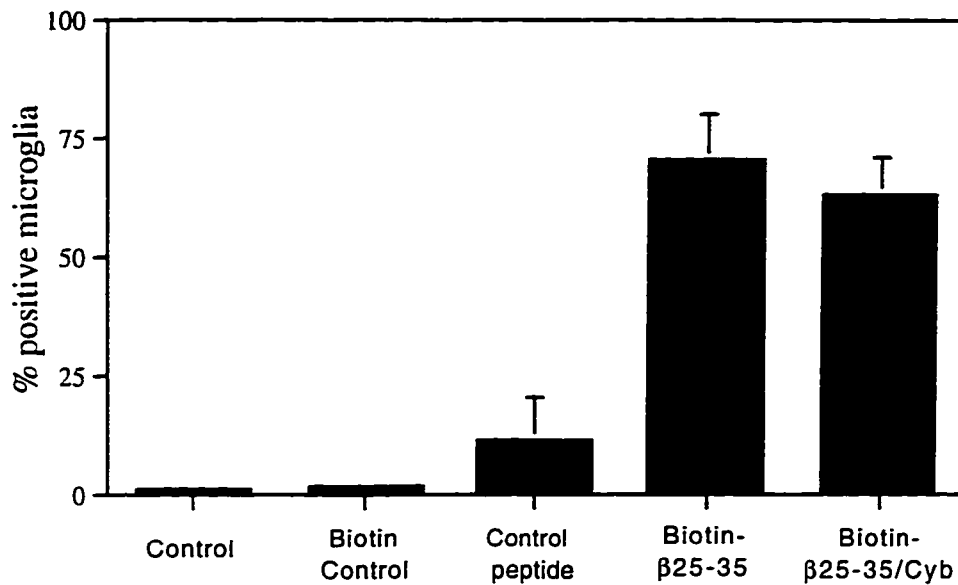


Fig. 3. Percent positive labeling of microglia exposed to biotinylated β 25-25 for 15 minutes followed by labeling with streptavidin PE. Biotinylated β -amyloid-exposed cells showed a significant increase in % positive labeling as compared to the biotin control suggesting binding of β -amyloid to the cells. Binding of biotinylated β -amyloid was not significantly reduced when microglia were treated with cytochalasin B (CyB) before and during exposure to β -amyloid. Values represent % positive cells (gated population) \pm SEM for triplicate experiments.

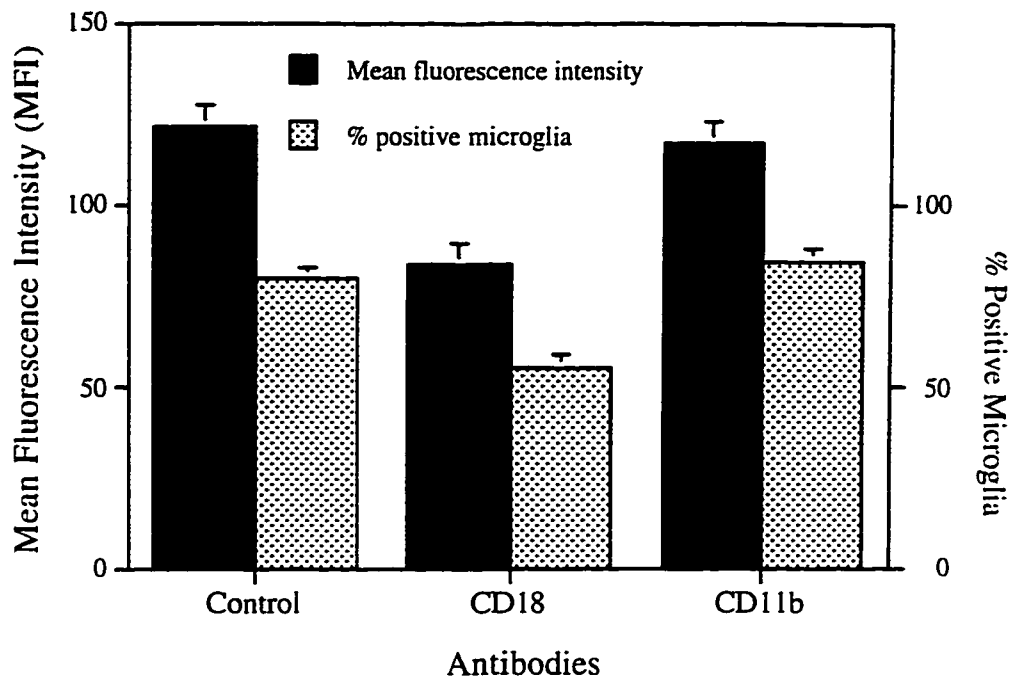


Fig. 4. Mean fluorescence intensity (MFI) and percent positive labeling of microglia exposed to biotinylated β 25-35 with and without preexposure to Wt.3 (anti-CD18 antibody) or OX42 (anti-CD11b antibody). Binding of biotinylated β -amyloid by microglia was significantly reduced by Wt.3, but not by OX42. Values represent the MFI or % positive cells (gated population) \pm SEM for triplicate experiments.

CHAPTER 4. GENERAL CONCLUSIONS

Summary and Discussion

The research in this dissertation was directed at an examination of the effect of β -amyloid on nitric oxide release from cultured microglia and a characterization of a mechanism by which β -amyloid might act to exert such an effect, i.e. via ligation of the Mac-1 receptor. The extent to which β -amyloid affects nitric oxide release from microglia was assessed in microglia exposed to two β -amyloid protein fragments (β 25-35 and β 1-40) alone and in combination with the co-stimulatory factors IFN- α/β , IL-1 β , TNF- α , TNF- β , and IFN- γ . The interaction of β -amyloid (β 25-35) and the microglial β_2 integrin receptor, Mac-1 was studied by quantifying the suppressive effects of anti-Mac-1 monoclonal antibodies (mAbs) on β -amyloid-mediated release of microglial nitric oxide and assaying competitive binding of biotinylated β 25-35 and anti-Mac-1 mAbs to microglial cells by immunofluorescent flow cytometry. Both sets of experiments generated positive and very interesting results.

In the first paper, microglial release of nitric oxide in response to β -amyloid was examined by exposing microglia to β 25-35 and β 1-40 coated on to the surface of culture wells. Assessment of microglial release of nitric oxide was based on the colorimetric assay for nitrite in the culture medium and histochemistry for nitric oxide synthase. Of the cytokines tested, only IFN- γ (1000 U/ml) induced nitric oxide release from microglia. β 25-35 did not stimulate nitric oxide release by itself, but it did induce nitric oxide release when co-exposed with IFN- γ (100, 500, 1000 U/ml). In contrast, β 1-40 did induce microglial release of nitric oxide by itself, and this effect was enhanced significantly by co-exposure with IFN- γ (100 U/ml). The results demonstrate that microglia are induced to release nitric oxide by β 1-40 alone and by the synergistic action of β 25-35 with IFN- γ . Almost identical findings have now been demonstrated, in part, by Meda, et.al.⁶ who reported a synergistic effect between β 25-35 and IFN- γ in triggering not only nitric oxide release, but tumor-necrosis factor- α (TNF- α) release

as well, from microglia. The morphological transformations induced by β -amyloid in our experiments (which have been described previously¹) and the increase in nitric oxide synthase activity, as well as nitric oxide release, induced by β -amyloid and IFN- γ as shown by our lab and the work by others, suggest that β -amyloid is an activator of microglia and may be responsible for activation of microglia with subsequent neuronal cell injury and degeneration *in vivo*. In the rodent, microglial nitric oxide release has been shown to be neurotoxic *in vitro*. The current findings support the view that oxidative damage may contribute to the process of neuronal degeneration that occurs in AD. It must be noted, as reviewed in the first paper, that there are interspecies differences in the production of nitrites from macrophages and microglia. Unfortunately, without comparable *in vivo* studies in humans, the assertion that microglial release of nitric oxide in response to β -amyloid plays a role in the pathogenesis of AD can be nothing more than speculation.

In search of a cellular binding site through which β -amyloid acts to trigger nitric oxide release from microglia, we investigated the leukocyte integrin Mac-1. In the second paper, the binding of β -amyloid with Mac-1, a cell surface receptor on microglia which has been histochemically shown to be upregulated in AD brains⁷, was studied by observing (1) β -amyloid (β 25-35)-mediated release of nitric oxide from cultured microglial cells following exposure to monoclonal antibodies against Mac-1 (anti-CD18 and anti-CD11b) and (2) competitive binding by microglia of fluorochrome-labeled β 25-35 and anti-CD18 or anti-CD11b by using fluorescent flow cytometry. Not surprisingly, Wt.3 (anti-CD18 antibody) and OX42 (anti-CD11b antibody) proved to be as effective as our positive control, opsonized zymosan, at inducing the release of nitric oxide from microglia. This is not the first report demonstrating monoclonal antibody activation of adhesion molecules⁵. Furthermore, Wt.3 and OX42 acted synergistically to induce maximum nitric oxide release. The results of this study demonstrate that Mac-1 mediates nitric oxide release in microglia. This is the first report which shows a connection between microglial Mac-1 and nitric oxide release.

An interaction between β -amyloid and CD18 of Mac-1 was evidenced, somewhat unconventionally, by the suppressive action of β 25-35 on Wt.3-mediated release of nitric oxide and the synergistic action between OX42 and β 25-35 in inducing nitric oxide release from microglia. The tissue culture studies were supported by a competitive binding assay of fluorochrome-labeled β 25-35 and Mac-1 antibodies (Wt.3 or OX42). Pre-exposure to Wt.3, but not OX42, significantly decreased binding of biotinylated β 25-35 to microglia. Taken together, the results of these studies suggest that Wt.3 and β 25-35 share a common site of action on the β chain (CD18) of Mac-1. The flow cytometry study revealed that the majority of microglial cells (71%) did bind biotinylated β -amyloid in the presence of Cytochalasin B, and this binding was upregulated by IFN- γ (100U/ml), all of which suggests that β -amyloid binding to microglia is a receptor-mediated event. The tissue culture studies suggest that β -amyloid binds to Mac-1, however the incomplete blocking effect of Wt.3 on microglial binding of β 25-35 raises some doubt as to the validity of this conclusion.

Two recent reports which suggest that there are other binding sites available that will bind to β 25-35 could explain the discrepancies in our results. As reviewed in the second paper, Yan et al.⁸ reported that β -amyloid binds with a microglia-surface receptor called RAGE (receptor for advanced glycation end products). An unrelated cell-surface receptor, class A SR (scavenger receptor), was also shown by El Khoury et al.² to interact with β -amyloid, leading to secretion of reactive oxygen species and cell immobilization. These studies suggest that RAGE and SR are involved in signaling microglia to accumulate at sites of β -amyloid deposition. They provide convincing evidence that microglia adhere strongly to β -amyloid fibrils which can result in microglial activation. However, a signal transduction pathway through which RAGE or SR can trigger the production of reactive mediators including reactive oxygen intermediates and nitric oxide has not been identified. It is possible that receptors such as RAGE and SR act in concert with Mac-1 which results in the pathological changes seen in AD. Perhaps β -amyloid in the plaques arrests cell migration via RAGE or SR which permits

microglia to undergo the sustained activation necessary to induce neurodegeneration via nitric oxide toxicity.

The hypothesis introduced in this dissertation, that the neurodegeneration in AD is the result of nitric oxide release from microglia in response to β -amyloid binding to microglial Mac-1, is not directed at the etiology, or first cause, of AD, but rather to a mechanism that may be involved in β -amyloid-mediated neurotoxicity. The findings reported here support the current 'amyloid theory' of AD by suggesting a route by which β -amyloid promotes neurodegeneration, in this case via microglial release of nitric oxide. If microglial nitric oxide release is proven to play a crucial role in AD, understanding the way in which β -amyloid affects microglial release of nitric oxide should be critical for studying the role of microglial cells in the pathogenesis of neuronal degeneration in AD. Subsequently, any microglial receptor known to bind to β -amyloid, i.e., RAGE, SR or Mac-1, may be possible targets for the development of drugs designed to reduce the chronic inflammatory component and neuronal injury associated with AD.

Recommendations for Future Research

We have shown that β -amyloid in combination with IFN- γ induces the release of nitric oxide from microglia via a Mac-1-mediated cell signaling pathway *in vitro*. It is important that we take our *in vitro* experiments one step further and attempt to induce neuronal toxicity in the culture dish via β -amyloid-mediated nitric oxide release from microglia. Inducing neuronal death by exposing neurons to microglia stimulated by β -amyloid and IFN- γ *in vitro* would further support a role for microglia in the pathogenesis of neurodegeneration in AD. The next logical step would be to reproduce these experiments in an *in vivo* model when an animal model becomes available. Progress towards an understanding of the pathogenic mechanisms at work in AD and in development and testing of therapies based on these mechanisms would be

helped by the availability of a convenient animal model. Two recent reports indicate that it may indeed be possible to mimic, in transgenic mice, significant aspects of the pathology in AD^{3,4}. Unfortunately, neither mouse model displays both major aspects of AD neuropathology, i.e. plaques and tangles. However, mice in which some of the pathologic changes of AD are seen may still be very useful for screening not only pathogenic mechanisms, but also potential drugs aimed at, i.e., blocking Mac-1-mediated activation of microglia and subsequent nitric oxide release in response to β -amyloid, or any other processes thought to play a role in the pathogenesis of Alzheimer's disease.

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ACKNOWLEDGEMENTS

The work described in this dissertation was accomplished with the help of a number of people. I would like to thank fellow graduate student Dr. Anja Parpura-Gill and laboratory technician Cathy Martens for their technical assistance, thoughtful criticism and overall support. I would like to thank my graduate professor, Dr. Etsuro Uemura, for his guidance and patience throughout this whole process. I cannot neglect to thank my family and my friends in Ames who provided that all-important emotional support. I would like to thank Susan Willard and again Cathy Martens and Dr. Etsuro Uemura for their assistance in the preparation of this dissertation. Finally, I would like to thank my early mentors, Dr. Maricio Pineda and Dr. Michael Doolen, who sparked my interest in research and set me on a path to what I hope will be a very gratifying career in Veterinary Medicine.